

Binding of ligands to the active site of carboxypeptidase A

(enzyme activity/proteases/protein crystallography/solid-state activity of enzymes/enzyme inhibition kinetics)

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ABSTRACT We compare the detailed binding modes of the 39-amino acid inhibitor from potatoes, glycyl-L-tyrosine, the ester analogue $\text{CH}_3\text{OC}_6\text{H}_4(\text{CO})\text{CH}_2\text{CH}(\text{CO}_2)\text{C}_6\text{H}_5$, and indole acetate to the exopeptidase carboxypeptidase A (EC 3.4.17.1). In the potato inhibitor, cleavage of the COOH-terminal glycine-39 leaves a new carboxylate anion of valine-38 having one oxygen on zinc and the other as a receptor of a hydrogen bond from tyrosine-248 of carboxypeptidase. Tyrosine-248 also receives a hydrogen bond from the amide proton of the originally penultimate peptide bond between tyrosine-37 and valine-38. This hydrogen bond suggests product stabilization which is available to peptides and decapeptides but not to esters lacking an equivalent peptide bond (non-specific esters). Also, this structure may represent the intermediate binding step for the uncleaved substrate as it moves along the binding subsites. In particular, this may be the binding mode for the substrate after association of the COOH-terminal region of the substrate with the residues at binding subsite S_2 (tyrosine-198, phenylalanine-279, and arginine-71) and preceding entry into the catalytic site S_1 . These stabilized complexes allow some understanding of the effect of indole acetate, shown here to bind in the pocket at S'_1 , as a competitive inhibitor for esters (for which entry into S'_1 precedes the rate-determining catalytic step for hydrolysis) and as a noncompetitive inhibitor for peptides (for which entry into S'_1 is rate limiting). These results, including the binding mode of the ester analogue, are consistent with the original proposal from x-ray studies that both esters and peptides are cleaved with the carboxy terminus at S'_1 , although not necessarily by the same chemical steps.

A complete description of the mechanism of an enzyme requires understanding of the detailed succession of binding and catalytic steps. The nature of the interactions between the enzyme and substrate as the substrate progresses through the binding groove and into the active site pocket may profoundly influence the observed binding and catalytic characteristics. The mechanism of the exopeptidase carboxypeptidase A (CPase A; peptidyl-L-amino-acid hydrolase, EC 3.4.17.1) has been discussed from this viewpoint (1). In this paper, we describe crystallographic studies of the binding of a ketonic substrate, competitive and non-competitive inhibitors, and product molecules to CPase A in relation to the progression of binding steps and the mechanisms of hydrolysis of peptides and esters.

A significant result for the mechanistic interpretation of CPase A inhibition studies is that noncompetitive and competitive inhibitors can bind to the same site on the enzyme. This observation is inconsistent with an interpretation of inhibition kinetics based on a simple Michaelis-Menten model in which noncompetitive and competitive inhibitors bind to distinct sites (2). For CPase A, the action of various inhibitors on peptidase and esterase activity is more consistent with a multistep mech-

anism in which the kinetic effects of an inhibitor depend on the details of the rate-determining step for a particular substrate.

MATERIALS AND METHODS

CPase A_α (Cox), glycyl-L-tyrosine (Gly-Tyr), and indole-3-acetic acid (IAA) were purchased from Sigma and used without further purification. (-)-2-Benzyl-3-*p*-methoxybenzoylpropionate (BMBP) was a generous gift of E. T. Kaiser. Crystals of CPase A were prepared and crosslinked as described (3). It must be stressed that the native CPase A crystals used for all crystallographic work (space group $P2_1$, unit cell parameters $a = 51.60$ Å, $b = 60.27$ Å, $c = 47.25$ Å, $\beta = 97.27^\circ$, with the crystal habit elongated along the a axis) exhibit one-third the activity of the enzyme in solution (4). These crystals are different from crystals of CPase A_γ (Anson) (space group $P2_1$, unit cell parameters $a = 50.9$ Å, $b = 57.9$ Å, $c = 45.0$ Å, $\beta = 94.67^\circ$, with the crystal habit elongated along the b axis), which only exhibit 1/300th of the activity of the enzyme in solution (5). The less-active form of the CPase A crystals generally has been used for biochemical studies (6). The distinction between these two forms must be recognized when comparisons of solution/crystal enzyme activities are discussed.

Crystal soaking conditions for the difference map studies of Gly-Tyr (7) and BMBP (3) binding to CPase A have been documented. Crystals of the CPase A-IAA complex were prepared by soaking crosslinked crystals of native CPase A in 10 mM IAA/0.1 M NaCl/20 mM Veronal, pH 7.5, for 48 hr. Crystallization conditions and structure determination of the complex between CPase A and the 39-amino acid inhibitor from potatoes (PCI) have been described (8). Especially relevant for this discussion, the CPase A-PCI complex was crystallized from solution in a trigonal crystal form that is unrelated to the crystal form of the native enzyme. Moreover, there are two molecules of this complex in different crystallographic environments. Thus, this crystal structure provides an independent view of the CPase A structure and CPase A-ligand interactions to complement the work on the native crystals.

Data collection and structure determination of the native enzyme (9) and of the complexes of CPase A with Gly-Tyr (7), BMBP (3), and PCI (8) at 1.75-, 2.0-, 2.8-, and 2.5 Å resolution, respectively, are described elsewhere. Coordinates for the native enzyme and the CPase A-PCI complex have been refined to crystallographic R factors of 0.174 and 0.196 at 1.5- and 2.5 Å resolution, respectively, by using the restrained least squares refinement algorithm of Hendrickson and Konnert (10) (unpublished data; M. Lewis, personal communication). A 2.3 Å resolution data set was collected from a single crystal of the CPase A-IAA complex on a Syntex P2₁ diffractometer at 20°C.

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Abbreviations: CPase A, carboxypeptidase A; IAA, indole-3-acetic acid; BMBP, (-)-2-benzyl-3-*p*-methoxybenzoylpropionate; PCI, potato carboxypeptidase A inhibitor; AAT, arsanilazotyrosine-248.

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Data processing and graphics procedures have been described (9).

Least squares estimates of the occupancies of the ligands in these complexes were obtained by the usual refinement procedures (11). An isotropic temperature factor of 12 \AA^2 was assumed for all ligand atoms. The ligand coordinates, obtained from the graphics program, were not refined. Populations of 43%, 54%, and 28% were obtained for the Gly-Tyr, IAA, and BMBP complexes with CPase A, respectively.

RESULTS

The active site region of CPase A in the native enzyme is illustrated in Fig. 1 for comparison with the CPase A-ligand complexes described below.

Binding of Gly-Tyr. The binding interactions of Gly-Tyr with CPase A (7, 9) are illustrated in Fig. 2. Gly-Tyr is a slowly cleaved substrate and a competitive inhibitor for more rapidly cleaved peptide substrates. The tyrosine ring of Gly-Tyr occupies the S'_1 binding pocket; the carboxylate group forms a salt link to arginine-145, and the peptide bond carbonyl oxygen binds to the zinc. Interestingly, the amino nitrogen of Gly-Tyr is statistically distributed between a coordination site on the zinc and a salt bridge to the carboxylate group of glutamate-270. In response to the binding of Gly-Tyr, several pronounced conformational changes occur in CPase A: the most prominent is the movement, by about 8 \AA , of the tyrosine-248 ring from the native "up" position to the "down" position, where the hydroxyl proton may interact with the carboxylate group of Gly-Tyr. Smaller but significant changes occur in the conformation of arginine-145, isoleucine-247, and glutamate-270. In addition, several water molecules are displaced upon binding of Gly-Tyr. The entropic effects associated with the release of bond water may play a significant role in the thermodynamics of ligand binding and catalysis.

Binding of BMBP. CPase A has been shown to catalyze a stereospecific exchange of one of the methylene protons of the ketonic substrate BMBP (12). The moderate rotational freedom around the ketone group suggests that BMBP should resemble an ester more closely than a peptide substrate. The major features of BMBP binding are similar to those of Gly-Tyr (3): the phenyl ring occupies the S'_1 binding site, the carboxylate group binds to arginine-145, and the carbonyl oxygen is coordinated to the zinc (Fig. 3). The *p*-methoxybenzoyl group binds near tyrosine-198. The methylene group, which undergoes the ster-

eospecific proton exchange, is located near glutamate-270.

Binding of IAA. IAA is a noncompetitive inhibitor of peptidase activity but a competitive inhibitor of esterase activity (13). The indole ring of IAA occupies S'_1 , and the carboxylate group binds to arginine-145 (Fig. 4). Only one binding site for IAA was observed at the concentration used in this experiment. In view of the differences in inhibitor activity of IAA and Gly-Tyr, it is important that, to within the limits imposed by the dissimilarities in molecular structures of the two ligands, IAA and Gly-Tyr bind to the same region of the enzyme.

Binding of PCI. The previous binding studies have been concerned with small ligands which bind to CPase A primarily at the S'_1 and Zn binding sites. The complex of CPase A with the 39-amino acid PCI molecule indicates the interaction of an extended substrate to CPase A (Fig. 5). The S'_1 , S_1 , S_2 , and S_3 sites are occupied by glycine-39, valine-38, tyrosine-37, and proline-36, respectively, in the CPase A-PCI complex (8). (In the following discussion, residue numbers below 40 will refer to the PCI molecule.) Because glycine-39 is hydrolyzed from the remainder of the inhibitor and is trapped in the S'_1 pocket, the CPase A-PCI structure represents a product complex.

The two atoms formerly joined by the cleaved peptide bond, the amino nitrogen of glycine-39 and the carbonyl carbon of valine-38, are separated by 3.3 \AA in this complex. The new carboxylate anion of valine-38 (S_1 binding site) has one oxygen on the zinc and the other as a receptor of a hydrogen bond from tyrosine-248. Tyrosine-248 is located in the "down" conformation, similar to that seen in the previously described complexes. Tyrosine-248 also receives a hydrogen bond from the amide proton of valine-38. The carboxyl group of glutamate-270 is more than 4 \AA from the carboxyl group of valine-38, precluding the occurrence here of a stable anhydride species. However, this does not rule out an anhydride intermediate during peptide bond hydrolysis.

The carbonyl oxygen of tyrosine-37 (S_2 binding site) is hydrogen bonded to arginine-71. In position S_3 , proline-36 binds to CPase A near the aromatic groups of tyrosine-198 and phenylalanine-279. The presence of the proline, and an intramolecular hydrogen bond in PCI between the carbonyl oxygen of proline-36 and the indole nitrogen of tryptophan-28, creates a bend in the PCI chain as it leaves the active site region of CPase A. Consequently, a fifth binding site (S_4), which was proposed on the basis of kinetic studies (14), could not be identified in this work.

Outside of the differences in the active site region expected

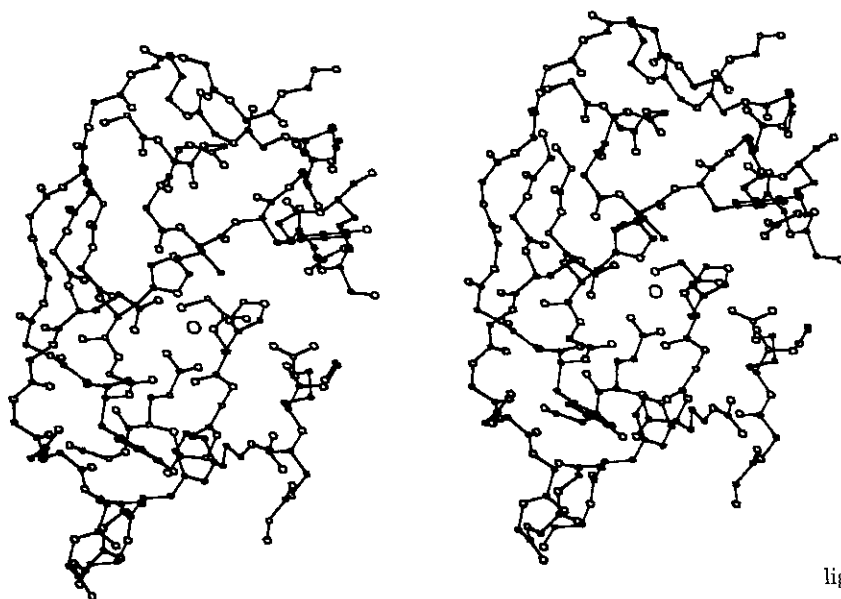


FIG. 1. Active site region of CPase A in the unliganded state.

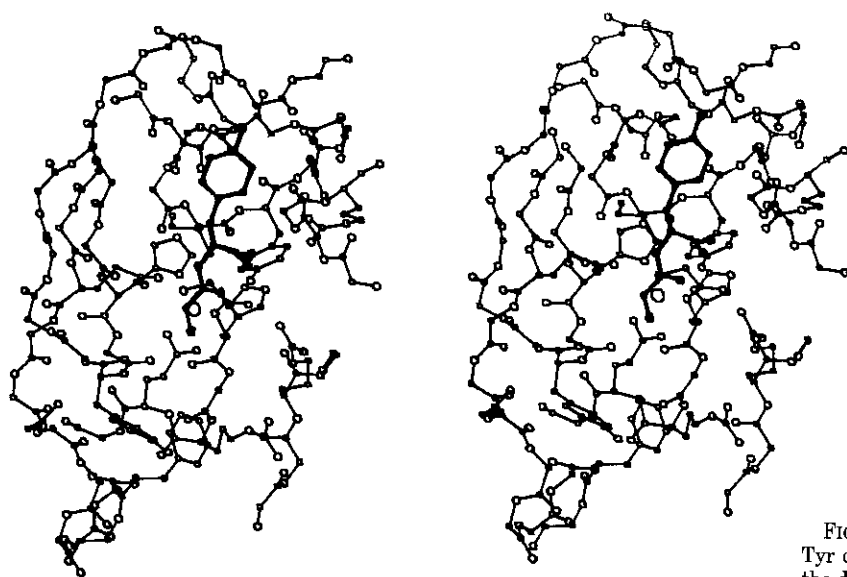


FIG. 2. Active site region in the CPase A-Gly-Tyr complex. The Gly-Tyr molecule is indicated by the darker bonds.

from earlier crystallographic studies, the structures of CPase A determined from the native and CPase A-PCI crystals are the same to within the limits of the resolution and refinement algorithms. The main chain coordinates of the three crystallographically independent CPase A molecules solved from native and CPase A-PCI crystals may be superimposed on one another with root mean square deviations between 0.26 and 0.42 Å. Because the lattice contacts are different in these two crystal forms, the influence of the crystal packing on the CPase A conformation is small. Consequently, there would appear to be little reason to suspect significant differences between the molecular structures of CPase A in solution and in crystals, although flexible side chains such as tyrosine-248 are particularly responsive to environmental changes, including chemical modification and binding of substrates and inhibitors.

DISCUSSION

Mechanistic Considerations. The structures of the complexes of Gly-Tyr, BMBP, IAA, and PCI with CPase A are consistent with the proposal that the productive binding of both esters and peptides places the carboxy-terminal side chain of the substrate in the S'_1 pocket; the bond to be cleaved is located between the S'_1 and S_1 sites (1). An alternate hypothesis, that esters and peptides are cleaved in different sites, was based on striking differences in binding and kinetic characteristics between ester and peptide substrates (13, 15). An essential assumption behind this alternate view was that competitive inhibitors bind in the same site as a substrate but noncompetitive inhibitors bind in different sites. The binding of Gly-Tyr and IAA described here show that such an assumption is invalid—both ligands bind in S'_1 , with the carboxylate group on arginine-145.

The present observations support instead a model in which peptides and esters are cleaved in the same site, consistent with a multistep reaction having different rate-controlling steps for esters and peptides (1, 16). A simple model, in which entry of the carboxy-terminal residue into the binding site pocket S'_1 precedes the rate-determining catalytic step for esters but is the rate-determining step for peptides, is in accord with most of the crystallographic and kinetic studies of CPase A (1). This differential behavior of peptides and esters would be primarily due to more facile distortion of ester compared to peptide bonds.

Aside from these similarities in binding, however, different detailed mechanisms for hydrolysis may apply to different substrates, even though the cleavage would occur between S'_1 and S_1 (1). Although the present crystallographic experiments cannot distinguish between the anhydride and general base mechanisms, and do not even eliminate the zinc-hydroxyl mechanism, they do permit inferences concerning structural aspects of possible hydrolytic intermediates (1). For example, we can regard the PCI structure as a complex with both product molecules positioned for resynthesis of the peptide bond and then consider the implications of this structure for the reverse reaction, peptide bond hydrolysis. Assume, for discussion, the general base pathway for peptide bond hydrolysis and assume that the carboxylate oxygen of valine-38 bound to the zinc is the carbonyl oxygen of the original peptide bond. One plausible catalytic sequence is as follows. The oxygen of the water molecule promoted by glutamate-270 attacks the carbonyl carbon of the scissile peptide bond. Tyrosine-248 donates a proton to the released amino nitrogen, leaving a phenolate anion. The orientation of tyrosine-248 in the CPase A-PCI complex suggests that this residue is now properly positioned to accept a proton from the attacking water molecule. Thus, the same water

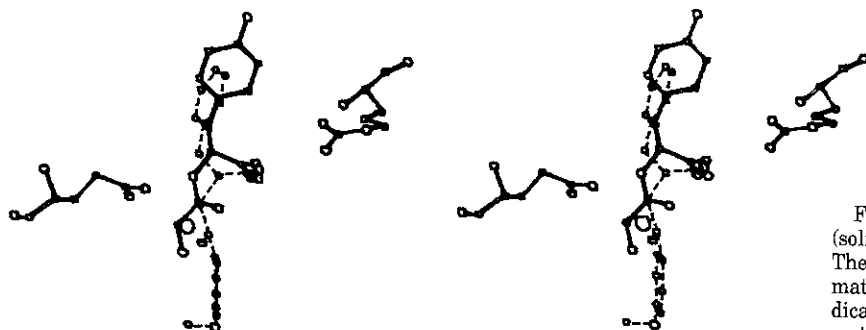


FIG. 3. Comparison of the binding of Gly-Tyr (solid lines) and BMBP (dashed lines) to CPase A. The conformations of Zn, arginine-145, and glutamate-270 in the CPase A-Gly-Tyr complex are indicated. The active site is viewed from the same direction as in Fig. 1.

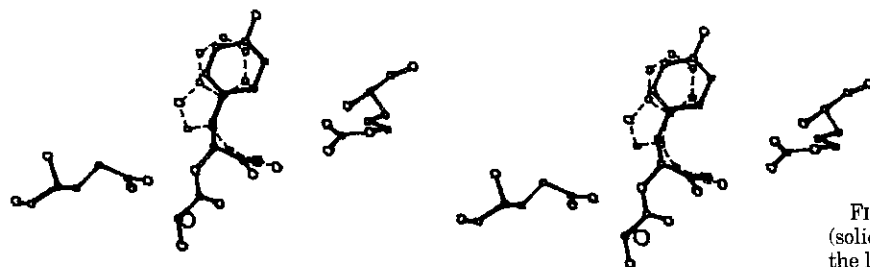


FIG. 4. Comparison of the binding of Gly-Tyr (solid lines) and IAA (dashed lines) to CPase A. See the legend to Fig. 3 for additional details.

molecule that attacks the peptide bond could also provide the proton to regenerate tyrosine-248, without requiring a second water molecule for this function. (One alternative, in which both glutamate-270 and Zn promote the attack of one water, would yield a newly formed carboxylate anion which has the carbonyl oxygen, originally on Zn, finally oriented toward tyrosine-248; proton transfer might then require additional steps.) The formation of a hydrogen bond between tyrosine-248 and the amide proton of the originally penultimate peptide bond between tyrosine-37 and valine-38 (Fig. 5) also suggests a product stabilization interaction for peptides and deipeptides. This hydrogen bond could not be formed for esters lacking an equivalent peptide bond (nonspecific esters), however, and may be responsible for some of the kinetic differences between these two classes of esters.

Another important mechanistic question concerns entry of the substrate into the active site pocket. The interaction of the valine-38 carboxyl group with the zinc may represent a penultimate mode of substrate binding, which follows association of the carboxy-terminal region of the substrate with residues at S_2 (arginine-71, tyrosine-198, and phenylalanine-279). This binding stage would immediately precede entry of the carboxy-terminal residue into the S'_1 pocket. Because glycine-39 is unnecessary for PCI binding to CPase A (17) it is possible that the CPase A-PCI structure actually represents a complex that is trapped in this intermediate binding stage and is unable to proceed to the true catalytic transition state due to steric interactions between CPase A and the remainder of the PCI molecule.

Role of Tyrosine in Catalysis. In all of the difference map studies of ligand binding to CPase A in native CPase A crystals, strong negative electron density is observed at the original position of tyrosine-248 and positive density is present for the down orientation of this residue. This observation requires that binding of ligands to CPase A induces the conformational change in tyrosine-248 from the up to the down conformation. An earlier assignment, due to errors in the phases, of 15–25% of tyrosine-248 liganded to Zn in the native CPase A crystals has been revised downward by our refinement at 1.5-Å resolution. The occupancy of a tyrosine ring placed in this putative position refines to a value of $-4\% \pm 10\%$ at 1.5 Å resolution. This im-

plies that a tyrosine-248-Zn interaction occurs in very low percentage, if at all, in the native enzyme. This result and the activity of the crystals used in the x-ray structure determination strongly suggest that tyrosine-248 is up in the unliganded enzyme and that binding of substrates induces the conformational change to the down position.

Spectroscopic studies of arsanilazotyrosine-248 CPase A (AAT-CPase A), however, have been interpreted as suggesting that a tyrosine-248-Zn interaction is an essential prerequisite for catalysis (6, 18–22), in direct contradiction to the conclusion from the crystallographic studies. Although AAT may chelate to the Zn in unliganded AAT-CPase A, it seems likely that this interaction is a property of the arsanilazo modification. Extrapolation of this behavior to the unmodified enzyme may be misleading. AAT is able to form a bidentate chelate to the zinc, in contrast to the unidentate coordination that unmodified tyrosine-248 must form. In addition, the arsanilic acid group, with both an aromatic ring and an anionic acid function, contains structural features of preferred substrates for CPase A, possibly facilitating binding of this group to the enzyme. Both of these effects will promote enhanced binding of AAT to the zinc, in comparison to unmodified tyrosine-248. The pK for the AAT-Zn interaction is 7.7, in the middle of the pH-activity profile for unmodified CPase A. Consequently, the pK for the unmodified tyrosine-248-Zn interaction, if it occurs at all, must be greater than 7.7, placing it in the range where it would not occur to a significant extent over most of the pH range for CPase A activity. In addition, the kinetics of the AAT-Zn interaction indicate that this association is slower than the rate of rapid substrate hydrolysis (1, 4), again suggesting that the tyrosine-248-Zn ligand is not involved in the catalytic mechanism of unmodified CPase A.

On the basis of resonance Raman studies of AAT-CPase A, it has been suggested that an intermolecular hydrogen bond between tyrosine-248 and a neighboring CPase A molecule in the crystal is responsible for the low activity of CPase A crystals (22). This interpretation needs to be tested for the crystals of CPase A, used in these experiments, but it is clearly not true for the crystals used in the x-ray studies. As shown in Fig. 6, the closest intermolecular contact of the tyrosine-248 phenol oxy-

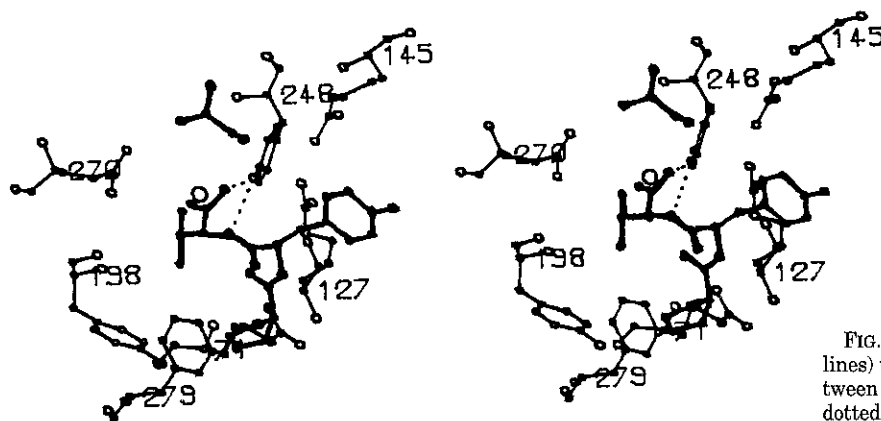


FIG. 5. Binding of residues 36–39 of PCI (thick lines) to the active site of CPA. Hydrogen bonds between tyrosine-248 and valine-38 are indicated by dotted lines.

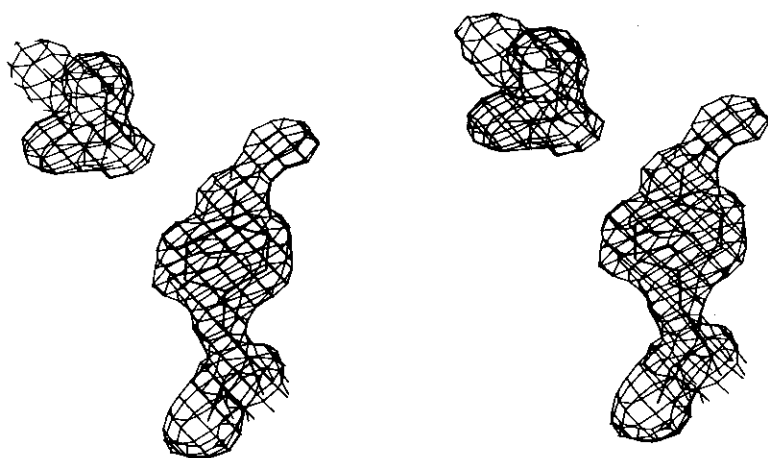


FIG. 6. Inter-molecular contacts of tyrosine-248 in the x-ray crystals. Threonine-14 and tyrosine-248 were omitted from the structure factor calculation from which this difference map at 1.5 Å resolution was drawn in order to obtain an unbiased electron density map. Coordinates for threonine-14 and tyrosine-248 are superimposed on the electron density.

gen is with the methyl group of threonine-14, 3.8 Å away. The phenol oxygen of tyrosine-248 is hydrogen bonded to a water molecule.

Studies on AAT-CPase A crystals also support the conclusion that tyrosine-248 has free mobility in the x-ray crystals. At pH 8.2, AAT-CPase A is red both in solution and in the x-ray crystals, indicative of an AAT-Zn interaction, whereas crystals of AAT-CPase A (Anson) are yellow, indicative of an unchelated AAT group (4). Based on comparison of the solution and crystal behavior of AAT-CPase A (Anson), Vallee and coworkers (6, 18–22) suggested that the solution and crystals conformations of CPase A are different. As shown by Quijcho *et al.* (4) however, the appropriate examination of the solution and crystal behavior of AAT-CPase A (Cox) demonstrated that the AAT groups are in similar environments in both states. These results once again stress the necessity of distinguishing between the various crystal forms of CPase A when interpreting experimental results.

Summary. We make the following observations.

(i) Because the crystals that we study show about one-third of the activity they have in solution, we have a functional enzyme in our crystals. The most readily available commercial crystalline form is less active by a factor of about 100.

(ii) Different sites for hydrolysis of peptides and esters are not required, provided entry into the active site pocket is rate limiting for peptides but precedes the rate-limiting catalytic steps for esters. Thus, IAA, which binds at S₁, can inhibit competitively for esters and noncompetitively for peptides. Of course, different detailed mechanisms may apply to members of these two classes even though cleavage occurs between S₁ and S₁' for both classes.

(iii) At pH 7.5, the percentage of tyrosine-248 anion-Zn complex in the absence of substrate is most probably zero and is certainly <10% in our 1.5 Å refinement. It is therefore likely that the arsanilazo tyrosine-248-Zn interaction is a property of this arsanilazo derivative of CPase A and that a tyrosine-248-Zn interaction occurs in low percentage, if it occurs at all, in the native enzyme. Activation of tyrosine-248 by Zn is therefore unlikely.

(iv) Inhibitors such as IAA might also bind noncompetitively to the enzyme in solution near tyrosine-198, phenylalanine-279, and arginine-71. Inter-molecular contacts in our crystal form may reduce binding near this "recognition" site.

(v) The binding occupancies of the ligands described in this paper are between 28% and 55%. Ligand binding to CPase A in our active crystals is accompanied by large negative electron

density at the original position of tyrosine-248 in the native enzyme. Thus, the binding of ligands to the enzyme induces the conformational change in tyrosine-248 described in the earlier x-ray diffraction studies.

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